FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
MIT-C102	09/889845

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/06174	05 NOV 99	10 FEB 99

TITLE OF INVENTION
CYCLIC PEPTIDES AND AIDS VACCINES

APPLICANT(S) FOR DO/EO/US
Shozo SHOJI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

FORM PCT/IB/308 - Notice Informing the Applicant of the Communication of the International Application to the Designated Offices

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/889845	PCT/JP99/06174	MIT-C102

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 - 20 =	0	x $18.00	**$0.00**	
Independent claims	3 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims (**check if applicable**).			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$860.00**	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$860.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$860.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (**check if applicable.** ☒				**$40.00**	
TOTAL FEES ENCLOSED =				**$900.00**	
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. _____ A duplicate copy of this sheet is enclosed.

d. ☒ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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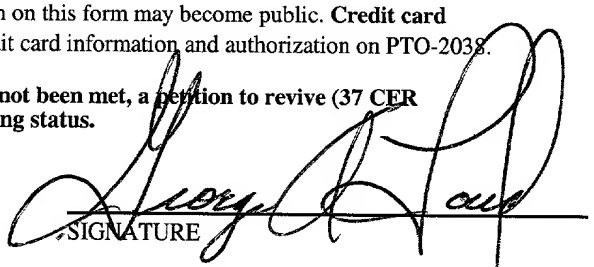

SIGNATURE

George A. Loud
NAME

25,814
REGISTRATION NUMBER

July 23, 2001
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

Shozo SHOJI

Serial No.:

Filed: July 23, 2001

For: CYCLIC PEPTIDES AND AIDS VACCINES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the captioned application as follows:

IN THE CLAIMS:

Please rewrite claim 4 as follows:

4. (Amended) Cyclic peptides as claimed in Claim1, wherein a substituent group is bonded to at least one active group selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic peptides.

Respectfully submitted,

George A. Loud
Reg. No. 25,814

Dated: July 23, 2001

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4. (Amended) Cyclic peptides as claimed in Claim 1 [Claims1, 2 or 3], wherein a substituent group is bonded to at least one active group selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic peptides.

SPECIFICATION

CYCLIC PEPTIDES AND AIDS VACCINES

FIELD OF THE INVENTION

The present invention relates to cyclic peptides effective in preventing HIV-1 virus infection in human and to AIDS vaccines. More particularly, it relates to cyclic peptides which serve as antigens for producing a neutralizing antibody capable of neutralizing HIV-1 virus infection via the second receptors called CXCR4 and CCR5 and to AIDS vaccines which comprise the above antigens as active ingredients.

BACKGROUND OF THE INVENTION

Second receptors which the pathogenic virus causative of AIDS (HIV-1 virus) utilizes in infecting human were identified in 1996 (Yu Feng et al., Science, 272, 872-877, 1996). These receptors are two receptors called CXCR4 and CCR5 among the chemokine receptors already reported. It has been revealed that the HIV-1 virus utilizes one of the receptors for adsorption onto and entry into lymphocytes, macrophages and dendritic cells to achieve infection.

On the other hand, about 1 to 2% of Caucasians reportedly have resistance to HIV-1 virus infection and it has been revealed that this is due to a genetic defect or genetic incompleteness of the second receptors (CXCR4 and CCR5), which are chemokine receptors (Rong Liu et al., 86, 367-377, 1996).

These findings have called researchers' attention to the importance of neutralization of the second receptors in the prevention of HIV-1 virus infection and, in recent years, attempts have been made to produce a neutralizing antibody capable of neutralizing the second receptors. There is no report, however, about the successful creation of such a neutralizing antibody.

Accordingly, it is an object of the present invention to provide three-dimensional antigens capable of producing, in vivo, a neutralizing antibody capable of neutralizing the second receptors from the stereoscopic viewpoint by paying attention to the loop structures of the second receptor proteins without following the conventional methods of interpreting the peptides constituting the second receptors two-dimensionally. Another object is to provide AIDS vaccines which comprise such antigens as active ingredients.

DISCLOSURE OF THE INVENTION

The present inventors constructed a model of the second receptor in T cells (abbr.: CXCR4) and a model of the second receptor in macrophages (abbr.: CCR5) and observed them from the three-dimensional viewpoint. As a result, they explored the applicability of two pentapeptides constituting the second subloop (UPL) in the respective second receptor proteins, namely T cell-derived Glu_{179}-Ala_{180}-Asp_{181}-Asp_{182}-Arg_{183} and macrophage-derived Ser_{169}-Gln_{170}-Lys_{171}-Glu_{172}-Gly_{173}, as constituent elements of a novel antigen for producing an HIV-1

virus infection-preventing antibody capable of neutralizing the second receptors and, as a result, they have now completed the present invention.

Thus, the present invention consists in a cyclic peptide which is a novel compound and comprises, as a constituent chain thereof, one or two amino acid sequences selected from among amino acid sequences contained in the second subloop in the T cell second receptor protein and comprising at least five amino acid residues and amino acid sequences contained in the second subloop in the macrophage second receptor protein and comprising at least five amino acid residues as well as in AIDS vaccines comprising that compound as active ingredients.

More specifically, the cyclic peptide of the present invention, which is a novel compound, is characterized in that it comprises one or two amino acid sequences selected from the groups consisting of the amino acid sequence Glu-Ala-Asp-Asp-Arg and the amino acid sequence Ser-Gln-Lys-Glu-Gly as a constituent chain or chains thereof, and the AIDS vaccine is characterized by comprising such compounds as active ingredients.

More particularly, the cyclic peptide of the invention is characterized in that it is a novel compound which is represented by the formula (1) given below and the AIDS vaccine of the invention is characterized in that it comprises that compound as an active ingredient.

Arg−Asp−Asp−Ala−Glu−Gly
 | | Formula(1)
Gly−Asp−Ser−Gln−Lys−Glu

Fig. 1 shows the configuration of a T cell-derived second receptor protein molecule on the T cell membrane (Fig. 1, top left) and the configuration of a macrophage-derived second receptor protein molecule on the macrophage membrane (Fig. 1, top right) and a cyclic dodecapeptide according to the invention as synthesized from the respective second subloop peptides of these second receptor protein molecules. In Fig. 1, the T cell-derived second receptor protein molecule (CXCR4) has a configuration comprising a first loop, a second loop, a third loop and a second subloop and the macropahge-derived second receptor protein molecule (CCR5) also has a configuration comprising a first loop, a second loop, a third loop and a second subloop.

The second subloop in the T cell-derived second receptor protein molecule (CXCR4) contains the amino acid sequence Glu_{179}-Ala_{180}-Asp_{181}-Asp_{182}-Arg_{183} and the second subloop in the macrophage-derived second receptor protein molecule (CCR5) contains the amino acid sequence Ser_{169}-Gln_{170}-Lys_{171}-Glu_{172}-Gly_{173}.

A novel compound cyclic dodecapeptide of the present invention as represented by the formula (1) shown above (cyclic peptide shown in Fig. 1, bottom) can be obtained by causing both the peptides respectively having the above-identified

amino acid sequences of both the second subloops of CXCR4 and CCR5 to form a ring via -Gly-Asp- as a spacer arm dipeptide.

Preferably, an active group selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic dodecapeptide represented by the above formula (1) is bonded to a substituent group so that the absorption into the living body and antibody formation may be facilitated. Such a substituent can be selected from among the residue of a fatty acid $CH_3(CH_2)_n$-COOH(n: 0 to 20), the residue of an alcohol $CH_3(CH_2)_n$-OH (n: 0 to 20) and the unsaturated compound residues corresponding to such compound residues and preferably has biocompatibility. As appropriate examples of the fatty acid, there may be mentioned a lauric acid, a myristic acid, a palmitic acid, a stearic acid, an arachidonic acid, and unsaturated fatty acids corresponding thereto. As appropriate higher alcohols, there may be mentioned a lauryl alcohol, a myristyl alcohol, a palmityl alcohol, a stearyl alcohol, an eicosanol, and unsaturated alcohols corresponding thereto.

The cyclic dodecapeptide represented by the above formula (1) can be utilized as an immunogen for producing a second receptor neutralizing antibody capable of inhibiting HIV-1 virus infection. In the following, mentioned is made of that immunogen.

An assaying antigen for antibody screening is prepared by binding the cyclic dodecapeptide to a solid phase resin. Separately, mice were immunized with an immunogen, for example a cyclic dodecapeptide-multiple antigen peptide (abbr.: CDP-MAP),

and monoclonal antibodies are prepared by the conventional hybridoma technique. For confirming the anti-infective activity against HIV-1 virus infection, several hybridomas (fused cells between antibody-producing B cells and myeloma cells (cancer cells)) are prepared by the above method and anti-HIV-1 virus activity assaying is carried out in the conventional manner using the hybridoma culture supernatants, whereby the culture supernatants prevent HIV-1 virus infection.

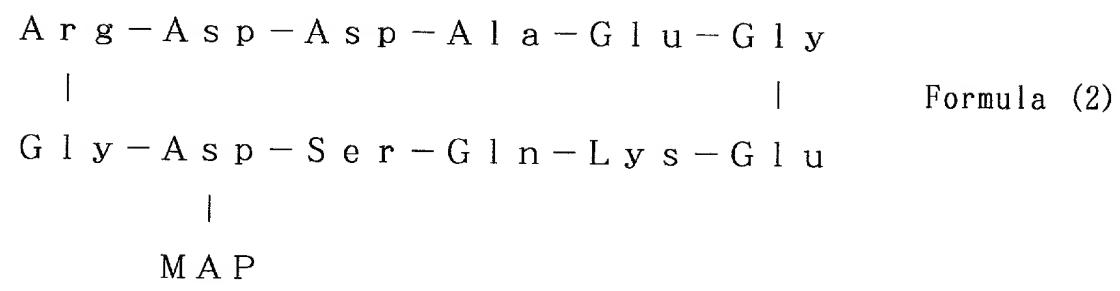
Thus, the cyclic dodecapeptide represented by the formula (1) can be used as an immunogen for producing antibodies having inhibitory effects against HIV-1 virus infection and therefore is useful as active ingredients in AIDS vaccines.

The AIDS vaccines according to the invention can comprise, as active ingredients, a cyclic peptide comprising, as a constituent chain or chains thereof, one or two amino acid sequences selected from the amino acid sequence Glu-Ala-Asp-Asp-Arg and the amino acid sequence Ser-Gln-Lys-Glu-Gly.

The AIDS vaccines according to the invention may comprise the above cyclic peptides as active ingredients or the active ingredients may be a modification derived from the cyclic peptides by substitution and/or addition or may be in the form of a pharmacologically acceptable salt. The pharmacologically acceptable salt includes salts with hydrochloric acid, a sulfuric acid, a nitric acid, a nitrous acid, a hydrobromic acid, a hydroiodic acid, a phosphoric acid and organic acids.

An example of the modification of the compound of the above formula (1) in which the substituent group is a higher fatty

acid group is shown below.



Formula (2)

Five equivalents of 9-fluorenylmethoxycarbonyl-dimethylsulfonium methyl sulfate (Fmoc-DSP; tradename, product of Novabiochem) are added to 1 equivalent of the cyclic dodecapeptide-MAP represented by the formula (2) to thereby block the ε-amino group of K_4 of the cyclic dodecapeptide-MAP and then the carboxyl groups (E_5, E_7, D_9, D_{10}) are activated with EDC, DCC, BOP or the like, and a higher alcohol [$CH_3(CH_2)_n$-OH] is added in excess to thereby effect esterification. Or, the hydroxyl group of Ser of the cyclic dodecapeptide-MAP represented by the above formula (2) is esterified by the acid chloride [$CH_3(CH_2)_n$ COCl] method and, after elimination of Fmoc, the ester is used as a base material of the peptide vaccine. When the vaccine is administered to the living body, it is delivered to lymphoid tissues, where the ester is hydrolyzed. The thus-recovered original cyclic peptide-MAP represented by the formula (2) activates the immune system, whereby antibodies are produced and the AIDS virus infection is neutralized.

The AIDS vaccines according to the invention can be used as a pharmaceutical compositions in the form or oral or nonoral

preparations. The oral dosage form includes tablets, powders, granules, capsules, microcapsules, solutions and the like. The nonoral or parenteral dosage form includes solutions, mainly injectable solutions, and suppositories, among others. Generally, these preparations may contain one or more of pharmaceutical preparation auxiliaries such as carriers, excipients, binders, disintegrants, lubricants, stabilizers, flavors, and the like.

The dose thereof may vary according to the symptom and/or age. In the case of oral administration, a daily dose of 0.1 to 1000 mg/kg body weight can be administered to ordinary adults.

BEST MODES FOR CARRYING OUT THE INVENTION

Example 1

(1) Synthesis of a cyclic chimera peptide comprising second subloop peptides of two types of receptors for HIV-1

The resin used for solid synthesis of the peptide was a 2-chlorotrisyl chloride resin, which will not impair the protective groups on various amino acid residues and from which the peptide can be cleaved with a weak acid. A 0.25-mmol (368-mg) portion of the resin was weighed and used. The peptide synthesis was carried out according to the Fmoc (9-fluorenylmethoxycarbonyl) chemistry and a Fmoc-side chain-protected peptide-resin was obtained by starting the synthesis from the C terminus on a fully automated peptide synthesizer using the following Fmoc-side chain-protected amino acids 1) to 12) (1.0 mmol each).

1) Fmoc-Gly-OH 1.0 mmol

2) Fmoc-L-Arg(Pmc)-OH 1.0 mmol

Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl

3) Fmoc-L-Asp(OtBu)-OH 1.0 mmol

OtBu: O-t-butyl

4) Fmoc-L-Asp(OtBu)-OH 1.0 mmol

5) Fmoc-L-Ala-OH 1.0 mmol

6) Fmoc-L-Glu(OtBu)-OH 1.0 mmol

7) Fmoc-Gly-OH 1.0 mmol

8) Fmoc-L-Glu(OtBu)-OH 1.0 mmol

9) Fmoc-L-Lys(Boc)-OH 1.0 mmol

Boc: benzyloxycarbonyl

10) Fmoc-L-Gln(Trt)-OH 1.0 mmol

Trt: trityl

11) Fmoc-L-Ser(tBu)-OH 1.0 mmol

tBu: t-butyl

12) Fmoc-L-Asp(OBzl)-OH 1.0 mmol

OBzl: O-benzyl

The protected peptide resin (300 mg) obtained in the above process was admixed with 5 ml of an acetic acid/trifluoroethanol/dichloromethane (1:1:8) mixture, the mixture was stirred at room temperature for 30 minutes and then filtered to thereby separate the side chain-protected peptide liberated with the weak acid from the resin, and ether was added to the filtrate in the conventional manner. To the thus-obtained precipitate was added an appropriate amount of acetonitrile, followed by lyophilization. By causing the carboxyl group of the C

terminal Gly of this side chain-protected dodecapeptide to condense with the amino group of the amino terminal Asp(OBzl) thereof, a cyclic dodecapeptide was synthesized as follows.

The side chain-protected linear dodecapeptide (130 mg) was dissolved in 80 ml of a dimethylformamide solution containing 10% trifluoroethanol, 5 times the amount of the peptide of benzotriazol-1-yloxytris(dimethylamino)phopshonium hexafluorophosphate (abbr.: BOP), the mixture was allowed to stand at room temperature for 24 hours to thereby allow the reaction to proceed, and 80 mg of a side chain-protected cyclic peptide was recovered by the conventional method.

This side chain-protected cyclic dodecapeptide was dissolved in 10 ml of dimethylformamide, 50 mg of palladium-carbon was added, catalytic reduction was carried out using hydrogen gas for 24 hours, and a carboxymethyl side chain-protected cyclic dodecapeptide (15 mg) was obtained by the conventional method. For identifying the cyclic dodecapeptide, all the protective groups were eliminated in the conventional manner and laser mass spectrometry was performed (MALDI-TOF mass spectrometer). The theoretical values and measured values for the cyclic peptide and linear (noncyclic) peptide are given below in Table 1. In Fig. 2, the MALDI TOF mass spectra for the cyclic peptide and linear (noncyclic) peptide are shown. The cyclic dodecapeptide was thus identified based on the results shown (reduction by molecular mass of water 18 as a result of dehydration condensation under ring formation).

Table 1

	Mass	Theoretical value	Measurement value
Cyclic peptide	1287.53	1288.53	1288.54
Linear (noncyclic) peptide	1305.54	1306.55	1306.73

(2) Preparation of immunogen comprising cyclic dodecapeptide-MAP (abbr.: CDP-MAP)

The carboxyl group of the carboxymethyl side chain-blocked cyclic dodecapeptide (abbr.: CM-SBCDP) was condensed with the amino group of tetra-branching polylysine of a MAP resin by the BOP method, as follows.

70 mg (32 μmol) of the MAP-resin (0.46 mmol tetra-branching polylysine/resin) was swelled in dimethylformamide (DMF) and the MAP-resin was deprotected (elimination of Fmoc) three times with 10 ml of 20% piperidine/dimethylformamide, washed three times with 5-ml portions of isopropanol and then deprived of the isopropanol, to expose the amino terminus of the tetra-branching polylysine. To this MAP-resin was added 10 ml (32 μmol) of a solution of the carboxymethyl side chain-blocked cyclic dodecapeptide in dimethylformamide and the binding between them was effected by the BOP method. The peptide was cleaved from the side chain-blocked cyclic dodecapeptide (abbr.: SBCDP)-MAP-resin in the conventional manner by treatment with trifluoroacetic acid (abbr.: TFA), whereby 12 mg of the

cyclic dodecapeptide-MAP (abbr.: CDP-MAP) was obtained. This was used as an immunogen for preparing anti-cyclic dodecapeptide (abbr.: Anti-CDP) monoclonal antibodies.

(3) Preparation of CDP-pin resin (crown resin) as assaying antigen for preparing anti-cyclic dodecapeptide (Anti-CDP) monoclonal antibodies

The assaying antigen for efficiently producing anti-CDP monoclonal antibodies from culture supernatants was prepared in the following manner. The side chain-blocked cyclic dodecapeptide was bound to β-Ala at the pointed end of the pin resin (crown resin) according to the epitope scanning kit manual (Chiron Mimotopes Pty Ltd, Clayton, Victoria, Australia) to give a CDP-pin resin (crown resin).

(4) Preparation of monoclonal antibody-producing hybridomas

Balb/c mice were primarily immunized using the cyclic dodecapeptide-MAP as the immunogen peptide and cell fusion was carried out in the conventional manner using myeloma cells (P3U1) and polyethylene glycol. After fusion, selective culture was carried out using HAT medium and, for the wells in which hybridoma cells formed colonies, the antibody titer in each culture supernatant was determined by the multi-pin ELISA method using the antigen peptide. For each cell group judged as antibody-positive, cloning was performed twice by limiting dilution and a monoclonal antibody-producing hybridoma line was established by the conventional method. For basal immunization, the lyophilized immunogen peptide was dissolved in PBS(-) to a concentration of 1 mg/ml and this solution was admixed, at a

ratio of 1:1.2 to 1:1.4, with the immunostimulator Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA), and the thus-prepared emulsion was used. This emulsion was intraperitoneally administered at a dose of 400 μl/mouse four times in total at one-week intervals. For the first two administrations, an emulsion with FCA was used and, for the last two administrations, an emulsion with FIA was used. The final or boost immunization was carried out after the lapse of one month following completion of the basal immunization by intravenous administration, through the caudal vein, of a 200μg/ml solution of the lyophilized immunogen peptide (MAP) in PBS(-) at a dose of 200 μl/mouse.

① Preparation of splenic cells and cell fusion

The preparation of splenic cells and cell fusion were carried out in the conventional manner. Three or four days after the final immunization, mice were sacrificed by exsanguination, splenocytes were excised and loosened in Hank's balanced salt solution (HBSS) and deprived of erythrocytes by hemolytic buffer treatment and centrifugation. The splenic cells thus prepared were mixed with P3U1 cells at a ratio of P3U1: splenic cells = 1:8 to 1:10 and the mixture was centrifuged. A polyethylene glycol solution was added to the pellet obtained to thereby effect fusion. After fusion treatment, the fused cells were gently suspended in HAT medium and the suspension was distributed in the wells of 48-well plates and cultured at 37℃ until the fused cells formed colonies.

② Screening for antibody-producing hybridomas

Screening for specific antibody-producing hybridomas was effected and the desired hybridomas were selected by continuously carrying out primary screening by the ELISA method using the immunogen peptide as a solid phase antigen and secondary screening using the multi-pin peptide as a solid phase antigen. In ELISA, the hybridoma culture supernatant was used as a primary antibody, peroxidase (POD)-labeled anti-mouse IgG as a secondary antibody, TMBZ (3,3',5,5'-tetramethylbenzidine) as a color substrate, and 0.3 N H_2SO_4 as color development stop solution, and the absorbances were measured at a dominant wavelength of 450 nm and at a reference wavelength of 630 nm.

③ Cloning of a desired antibody-producing hybridoma line

A monoclonal hybridoma strain showing high antibody titer in the screening assay was subjected to limiting dilution to one cell/well. The thus-cloned cells were distributed, together with feeder cells prepared from the murine thymus, into the wells of 96 well plates and cultured. After two repetitions of this cloning procedure, the group of monoclonal cells was subjected to screening by multi-pin ELISA using the antigen peptide. The cell line which showed the highest antibody titer in both ELISA screenings was selected as the monoclonal antibody-producing hybridoma line and the monoclonal antibody was purified from the culture supernatant thereof in the conventional manner. The subclass of this monoclonal antibody was found to be IgM κ. This hybridoma was deposited on February 3, 1998 with the Agency of Industrial Science and Technology National Institute of Life Science and Human

Technology under the accession number FERM P-17198 and this deposition was transferred on October 27, 1998 to the international deposition under the Budapest Treaty under the accession number FERM BP-6925. The cell line established was extended and cultured and the cells were frozen stored in a liquid nitrogen tank.

(5) Anti-HIV activity assay

The anti-HIV activity was measured by the method of Maeda et al. (Y. Maeda, et al., 12th World AIDS Conference Geneva, Abstract P4, June 28-July 3, 1998). The culture fluid of the anti-CDP monoclonal antibody-producing cells created by the present inventors and that of the corresponding non-antibody-producing cells as a control as obtained under the same conditions were used. The antibody-containing culture fluid (200 μl) reduced the rate of infection with HIV-1 virus to 61% in 30 minutes and to 35% in 60 minutes as compared with the control and thus was established that it inhibits the infectivity of HIV-1 virus.

INDUSTRIAL APPLICABILITY

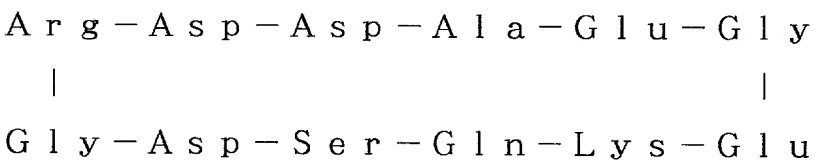
The cyclic peptide of the invention is a novel compound and is useful as an antigen for producing, in vivo, a neutralizing antibody (antibody having an anti-HIV-1 virus activity) capable of neutralizing the HIV-1 virus infection via the second receptor called CXCR4 and/or CCR5. It is also useful as an active ingredient of an AIDS vaccine.

CLAIMS

1. Cyclic peptides which comprise, as a constituent chain or chains thereof, one or two amino acid sequences selected from the groups consisting of the amino acid sequences comprising at least 5 amino acid residues as contained in the second subloop in the T cell-derived second receptor protein and the amino acid sequences comprising at least 5 amino acid residues as contained in the second subloop in the macrophage-derived second receptor protein.

2. Cyclic peptides which comprise, as a constituent chain or chains thereof, one or two amino acid sequences selected from the group consisting of the amino acid sequence Glu-Ala-Asp-Asp-Arg and the amino acid sequence Ser-Gln-Lys-Glu-Gly.

3. A cyclic peptide represented by the formula:



4. Cyclic peptides as claimed in Claims 1, 2 or 3, wherein a substituent group is bonded to at least one active group selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic peptides.

5. Cyclic peptides as claimed in Claim 4, wherein the substituent group is selected from among the residue of a fatty acid $CH_3(CH_2)n$-COOH (n: 0 to 20), the residue of an alcohol $CH_3(CH_2)n$-OH (n: 0 to 20) and the unsaturated compound residues corresponding to those compound residues.

6. AIDS vaccines which comprise the cyclic peptides according to Claim 1 as an active ingredient.

7. AIDS vaccines which comprise the cyclic peptide according to Claim 2 as an active ingredient.

8. An AIDS vaccine which comprises the cyclic peptide according to Claim 3 as an active ingredient.

ABSTRACT

Cyclic peptides comprising, as a constituent chain or chains, one or two amino acid sequences selected from the groups consisting of the amino acid sequence Glu-Ala-Asp-Asp-Arg and the amino acid sequence Ser-Gln-Lys-Glu-Gly, and AIDS vaccines containing the cyclic peptide as an active ingredient. Preferably a cyclic dodecapeptide represented by the formula given below and an AIDS vaccine containing the cyclic dodecapeptide as an active ingredient. From the in vivo absorption and antibody formation viewpoint, active groups selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic peptide is preferably bound to substituent groups. The cyclic dodecapeptide can neutralize the second receptors in the infection of human with HIV-1 virus.

Arg－Asp－Asp－Ala－Glu－Gly
　|　　　　　　　　　　　　|
Gly－Asp－Ser－Gln－Lys－Glu

Fig. 1

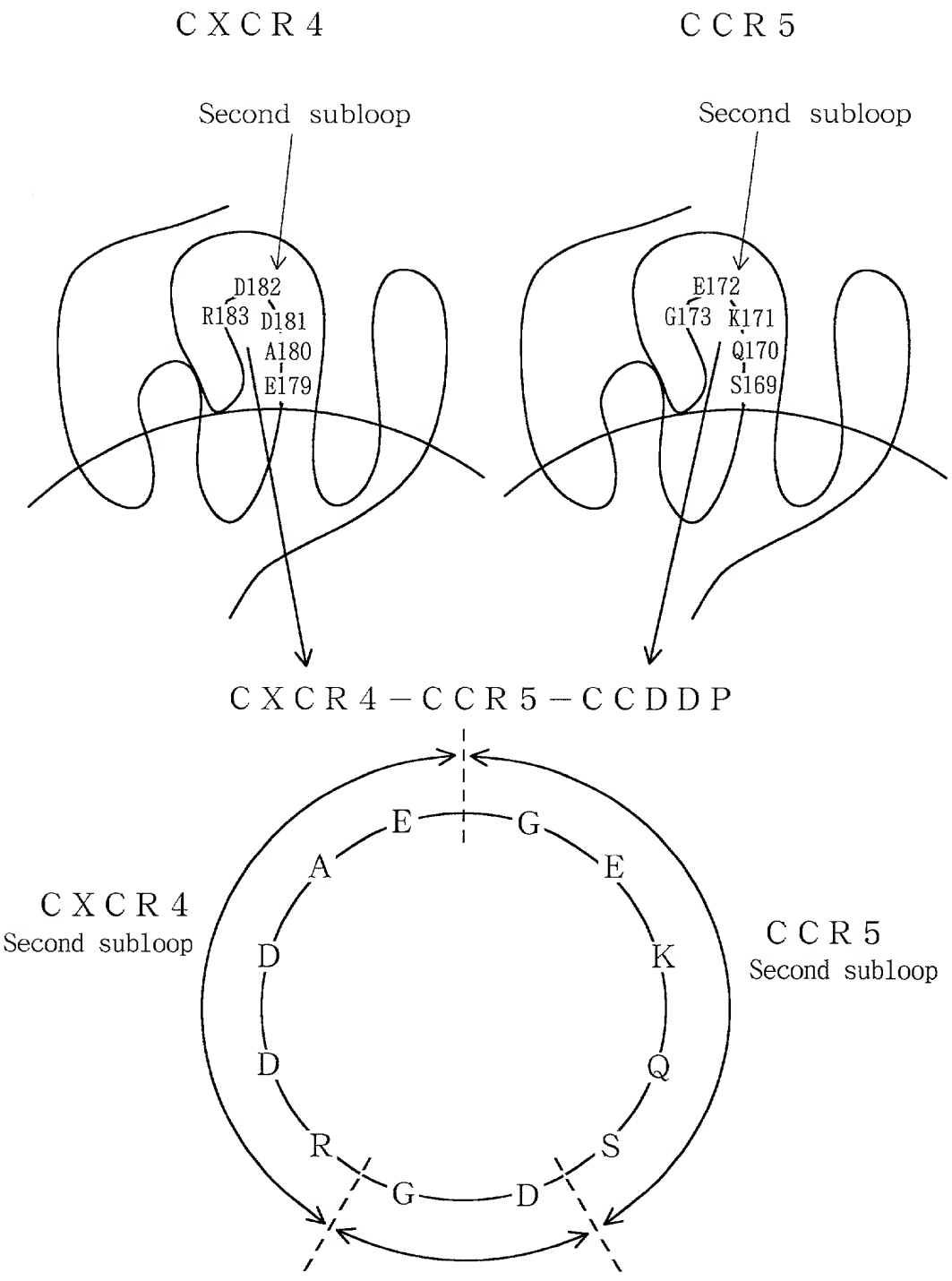
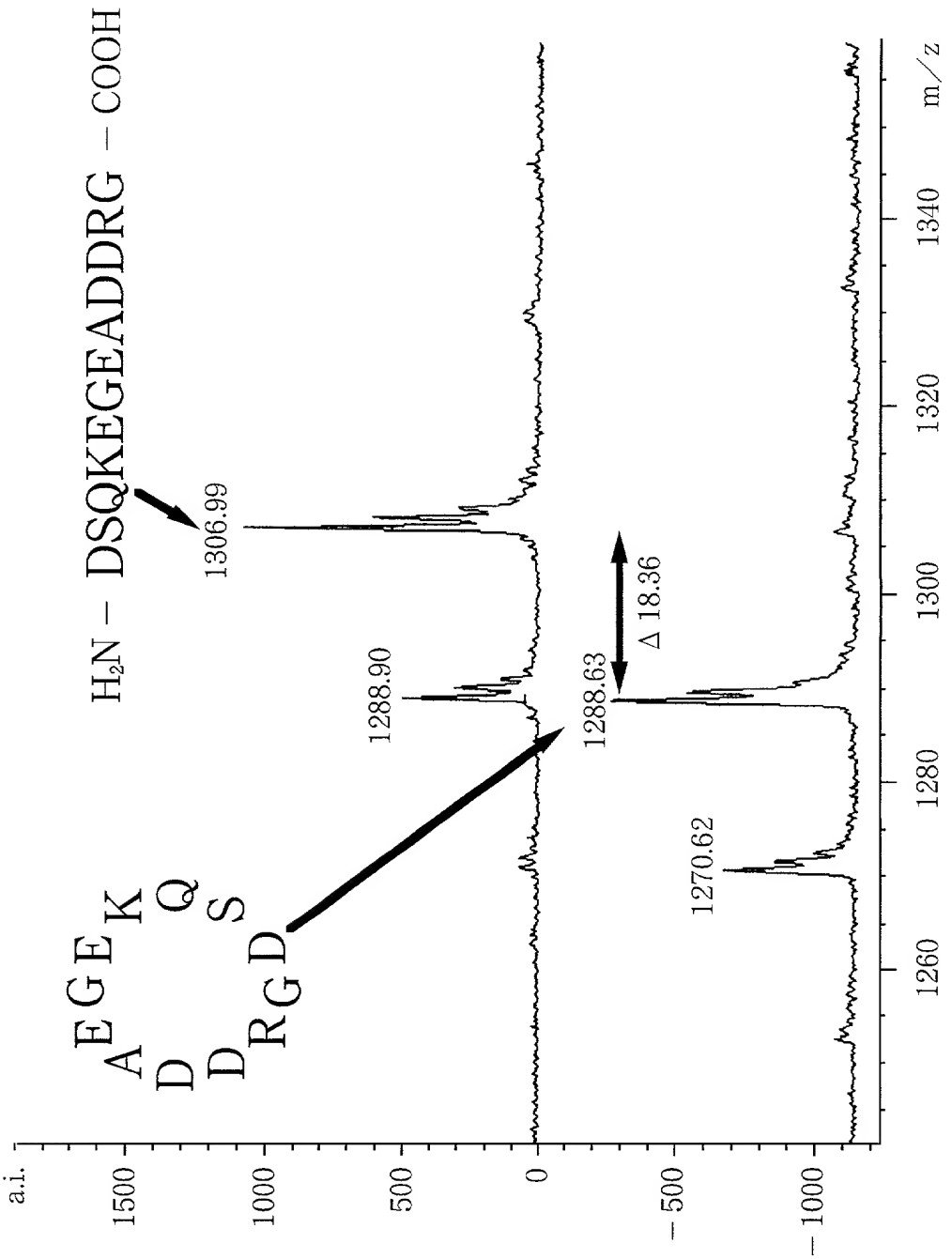

CXCR4
CCR5
Second subloop
Second subloop
D182
R183
D181
A180
E179
E172
G173
K171
Q170
S169
CXCR4－CCR5－CCDDP
CXCR4
Second subloop
CCR5
Second subloop
E
G
A
E
D
K
D
Q
R
S
G
D

Fig. 2


H2N – DSQKEGEADDRG – COOH
1306.99
1288.90
1288.63
Δ 18.36
1270.62
a.i.
1500
1000
500
0
– 500
– 1000
1260
1280
1300
1320
1340
m/z

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This declaration is of the following type:

[ ] original
[ ] design
[ ] supplemental
[X] national stage of PCT
[ ] divisional
[ ] continuation
[ ] continuation-in-part (CIP)

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and for which a patent is sought on the invention entitled:

CYCLIC PEPTIDES AND AIDS VACCINES

the specification of which

[ ] is attached hereto
[ ] was filed on _____, as Application Serial No. _____ and was amended on _____ (if applicable)
[X] was described and claimed in PCT International application No. PCT/JP 99/06174 filed on 05, November 1999 and as amended under PCT Article 19 on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any Amendment referred to above.

I acknowledge duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

I hereby claim foreign priority benefits under Title 35, United States Code, Sec. 119, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent of inventor's certificate having a filing date before that of the application on which priority is claimed:

[ ] no such applications have been filed
[X] such applications have been filed as follows.

Prior Foreign Application(s)

11-32990 (Number)	Japan (Country)	10 / February / 1999 (day/month/year filed)	[X] Yes	[ ] No
(Number)	(Country)	(day/month/year filed)	[ ] Yes	[ ] No

I hereby claim the benefit under Title 35, United States Code, Sec. 120 of any United States application(s) listed below, and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Sec. 112, I acknowledge the duty to disclose all information known to be material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Residence ______

Citizenship ______

Post Office Address ______

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